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AUTHOR(S):

Kakizawa, Sho; Shibazaki, Masahiko; Mori, Nozomu

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Protein oxidation inhibits NO-mediated signaling pathway for synaptic plasticity

Sho Kakizawa ^{a,b,*}, Masahiko Shibazaki ^a, Nozomu Mori ^{a,*}

^aDepartment of Anatomy and Neurobiology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan, ^bDepartment of Biological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

*Corresponding authors. Tel: +81 75 753 4552; fax: +81 75 753 4562.

E-mail address: sho-kaki@pharm.kyoto-u.ac.jp (S. Kakizawa) or morinosm@nagasaki-u.ac.jp (N. Mori).

Abstract

Oxidative stress is a primary factor inducing brain dysfunction in aged animals. However, how oxidation affects brain function is not fully understood. Here we show that oxidation inhibits signaling pathways essential for synaptic plasticities in the cerebellum. We first revealed that nitric oxide (NO)-dependent plasticities at parallel fiber–Purkinje cell synapse (PF synapse) were impaired in the cerebellar slices from aged mice, suggesting possible inhibitory action of protein oxidation by endogenous reactive oxygen species. PF-synaptic plasticities were also blocked in the cerebellar slices from young mice preincubated with oxidizing agents or thiol blocker. Because the treatment of the slices with the oxidizing agent did not affect basic electrophysiological properties of PF-EPSC and did not occlude the synaptic plasticities, oxidation was revealed to specifically inhibit signaling pathways essential for the PF-synaptic plasticities. Finally, biochemical analysis confirmed the idea that inhibitory action of protein oxidation on the PF-synaptic plasticities was mediated by impairment of NO-induced protein S-nitrosylation. Therefore, oxidation was revealed to inhibit S-nitrosylation dependent signaling pathway essential for synaptic plasticity in a “competitive” manner.

Keywords: aging, S-nitrosylation, oxidative agent, post-tetanic potentiation, long-term potentiation, cerebellum, Purkinje cell, parallel fiber

1. Introduction

Aging is associated with a general decline in physiological function in biological systems including the nervous systems (Barnes, 1988, 2003; Finch, 2003; Landfield, 1988; Mattson and Magnus, 2006). With the passage of time, the redox environment of the brain can be altered in favor of oxidation by an increased production of reactive oxygen species (ROS) or by a decreased activity of antioxidant defenses. This condition, known as oxidative stress or oxidative damage, is thought to be a general contributing factor to aging in the nervous systems (Beckman and Ames, 1998; Finkel and Holbrook, 2000; Harman, 1956). Actually, numerous studies have demonstrated various correlations between age and the accumulation of oxidative damage to cellular macromolecules (Floyd and Hensley, 2002; Stadtman, 2001). For example, enhanced lipid peroxidation (Calabrese et al., 2004; Devi and Kiran, 2004; Gupta et al., 1991; Murray and Lynch, 1998; O'Donnell and Lynch, 1998) and protein oxidation (Cini and Moretti, 1995; Forster et al., 1996; Sohal et al., 1994; Sultana et al., 2009; Vaishnav et al., 2007) are observed in the brains of aged rodents. Furthermore, several studies have shown that behavioral deficits of aged animals are associated with increases in oxidative stress (Butterfield et al., 2006; Cantuti-Castelvetri et al., 2000; Carney et al., 1991; Forster et al., 1996; Fukui et al., 2001). Although these associations between oxidative damage and brain dysfunctions cannot establish a causal link between the two, they do support the idea that oxidation are involved in

age-related brain dysfunction (Droge and Schipper, 2007; Serrano and Klann, 2004).

One type of cellular process strongly affected by oxidation is synaptic plasticity, a cellular process proposed as a biological substrate for learning and memory (Bliss and Collingridge, 1993; Ito, 2001; Lynch, 2004; Malenka and Nicoll, 1999). However, examination of the effects of oxidation on synaptic plasticity in studies where ROS (e.g. hydrogen peroxide) was applied exogenously to hippocampal slices resulted in paradoxical effects (Klann and Thiels, 1999; Serrano and Klann, 2004). Some studies suggest that ROS are essential for long-term potentiation (LTP) in hippocampal slices (Kamsler and Segal, 2003a; Knapp and Klann, 2002), whereas inhibitory effects of ROS are reported in other studies (Auerbach and Segal, 1997; Kamsler and Segal, 2003a, b; Pellmar et al., 1991; Watson et al., 2002). Different protocols were used for induction of synaptic plasticity and species or age of animals were different among these studies, and target signaling pathways of oxidation during the induction of synaptic plasticity were unclear in these experiments. Therefore, the identification of the target signaling systems of oxidation in synaptic plasticity would provide critical insight concerning how oxidative stress results in deficits in synaptic plasticity and brain function in aged animals.

Parallel fiber – Purkinje cell synapse (PF synapse) in the cerebellum is a good model for examining molecular mechanisms in synaptic plasticity in the central nervous systems (Hansel et al., 2001; Ito, 2006). Purkinje cells (PCs),

a solely output from the cerebellar cortex, receive two types of excitatory inputs: climbing fiber (CF) from inferior olive and PF, the axon of granular cells in the cerebellar cortex. Various types of synaptic plasticity were reported so far at PF synapse (Evans, 2007; Ito, 2006; Jorntell and Hansel, 2006). Among them, the synaptic potentiation induced by repetitive activity of PF are known to be dependent on nitric oxide (NO)-mediated signaling pathways (Kakegawa and Yuzaki, 2005; Lev-Ram et al., 2002; Namiki et al., 2005). Nitric oxide exerts its effects via two pathways. One pathway is mediated by soluble guanylyl cyclase (sGC). Activation of sGC induces increase in cytosolic cyclic GMP level and activates protein kinase G. Another pathway is mediated by S-nitrosylation of cysteine residues in various proteins. S-nitrosylation of proteins resulted in modification of function of proteins including ion channels and enzymes (Calabrese et al., 2007; Hess et al., 2005; Jaffrey et al., 2001; Nakamura and Lipton, 2007). Because the potentiation of PF synapse is dependent on NO but not sensitive to 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a specific antagonist for sGC, S-nitrosylation-mediated pathway is indicated to be involved in the potentiation (Lev-Ram et al., 2002; Namiki et al., 2005). Oxidizing agents including endogenous ROS also modify cysteine residues and exert its action on proteins (Ansari et al., 2006; Forman et al., 2008; Mikkelsen and Wardman, 2003; Suzuki et al., 2010). Therefore, NO and oxidizing agents competitively share cysteine residues to exert their action, and it is highly possible that oxidizing agents affect S-nitrosylation mediated signaling pathways essential

for the potentiation of PF synapse and impair or occlude the potentiation.

In the present study, we first demonstrated that post-tetanic potentiation (PTP) and long-term potentiation (LTP) at PF synapse were severely impaired in the cerebellar slices from aged (20 to 24-month old) mice, suggesting involvement of endogenous ROS in age-dependent decline in PF-PTP and PF-LTP. This hypothesis was confirmed by subsequent experiments demonstrating application of oxidizing agents also inhibited the induction of PF-PTP and PF-LTP in the cerebellar slices from young (4-6 weeks old) mice. Because both PF-PTP and PF-LTP is suggested to be dependent on S-nitrosylation, we propose that oxidation by ROS induce its inhibitory effects on synaptic plasticity via modification of cysteine residues whose S-nitrosylation by acute NO signal is essential for the induction of the plasticities, at least at PF synapses in the cerebellum.

2. Materials and methods

2.1. *Slice preparation*

All experiments were carried out according to the guidelines established by the Animal Welfare Committee of Nagasaki University.

Wild-type C57BL/6 mice at 4- to 6-weeks old or 20- to 24-months old were sacrificed by cervical dislocation under deep anesthesia with diethyl ether. The cerebellum was excised, and parasagittal cerebellar slices (250 μ m thick)

were prepared from the vermis (Edwards et al., 1989; Kakizawa et al., 2000, 2005). Whole-cell recordings were obtained from visually identified PCs under an upright microscope (BX51WI, Olympus, Tokyo, Japan) using a 40x water-immersion objective at room temperature (23-25°C). The resistances of patch pipettes were 2.0-3.5 M Ω when filled with an intracellular solution composed of (in mM), 130 K-gluconate, 10 KCl, 10 NaCl, 1 EGTA, 4 ATP-Mg, 0.4 GTP-Na and 10 HEPES (pH 7.3; adjusted with KOH). The standard bathing solution was composed of (in mM) 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, and 20 glucose, bubbled with 95% CO₂ and 5% CO₂. Antagonist for GABA_A receptor, bicuculline (10 μ M), was always added to block inhibitory postsynaptic currents.

2.2 Electrophysiology

For the focal stimulation of PF, a stimulation pipette (5-10 μ m in the tip diameter) was filled with the standard bathing solution and used in applying square pulses (0.1 ms in duration, 0-20 V in amplitude) in the molecular layer at the middle one-third from the pial surface. The intensity of each stimulus was adjusted to evoke PF-EPSCs with amplitude of 70–150 pA. Ionic current was recorded from PCs with a patch-clamp amplifier (EPC-9, HEKA, Lambrecht/Pfalz, Germany) at a holding potential of –90 or –80 mV, after the compensation of liquid junction potential. The signals were filtered at 2 kHz and digitized at 20 kHz. On-line data acquisition and off-line data analysis

were performed using PULSE (HEKA) software. Synaptic potentiation was induced by repetitive burst stimulation (BS) to PF (60 BS at 1 Hz; each BS consists of 5 pulses at 50 Hz) after the acquisition of baseline responses (Kakizawa et al., 2007; Namiki et al., 2005). Test stimulus was applied to PF every 10 s. The amplitude of PF-EPSC was averaged every 60 s, and normalized to the mean value observed for 10 min before the BSs. A 100-ms, -5 -mV hyperpolarizing test pulse preceded each PF stimulus to monitor the series resistance and input resistance of PCs throughout the experiment, the data of which were discarded if the resistance changed by more than 10% (Kakizawa et al., 2007; Namiki et al., 2005). The data were also discarded when the slope of PF-EPSC amplitude averaged every minute during the initial recording for 10 min was larger than 2% or when the amplitude did not become stable within 20 min after the onset of whole-cell configuration (Kakizawa et al., 2007; Namiki et al., 2005).

2.3 Application of oxidizing agents and thiol blocker

To examine the effects of preincubation of cerebellar slices with oxidizing agents on PF-synaptic plasticities, the slices were transferred to another incubation chamber, and solution containing hydrogen peroxide (H_2O_2) or chloramine-T (ChT) was directly added to the incubation media. The final concentration of H_2O_2 and ChT in the chamber were 1-100 μM and 0.1-10 μM , respectively. After the 10-minutes incubation with H_2O_2 or ChT, the slices

were transferred back to the original incubation chamber and kept for more than 1 hour until the electrophysiological analysis. In another experiment, cerebellar slices were preincubated with 50 μ M N-ethyl-maleimide (NEM) for 5 min. To examine the acute effects of the oxidizing agent on the amplitude of PF-EPSC, extracellular bathing solution was replaced with those containing 100 μ M H₂O₂ for 10 min. Flow rate of the perfusion system was 0.67 ml/min.

2.4. Biotin-switch assay for protein S-nitrosylation

Effects of aging and oxidizing agent on NO-induced protein S-nitrosylation in the cerebellar slices were examined by biotin-switch assay, the procedure being described by Forrester et al. (2007). This assay was performed in the dark. Cerebellar slices (about 100 μ g) treated with or without NO donor, NOC7, were homogenized with 200 μ l of lysis buffer (25 mM HEPES pH 7.7, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM Neocuproine, 0.1% NP-40, 1 mM PMSF). After centrifugation, 100 μ l of lysates were used for biotin-switch assay. Free cysteines were blocked for 1 h at 50°C in two volumes of HEN buffer (250 mM HEPES pH 7.7, 1 mM EDTA, 0.1 mM Neocuproine) containing 2.5% SDS and 0.1% methanethiosulfonate. Proteins were acetone precipitated at -20°C and resuspended in 240 μ l of HEN buffer containing 1% SDS. After adding fresh ascorbic acid (final concentration: 100 mM) and 60 μ g of EZ-link biotin-HPDP (Pierce), proteins were incubated at room temperature for 1 h. Subsequently,

proteins were acetone precipitated again and resuspended in 100 μ l of 0.1x HEN buffer containing 1% SDS. Then 100 μ l of 2x SDS-PAGE loading buffer (0.1M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol) were added and 20 μ l were used for 10% SDS-PAGE separation. After blotting onto Hybond-P membrane (Amersham), biotinylated proteins were detected with streptavidin-HRP (Amersham). Monoclonal antibody TUB2.1 (Sigma-Aldrich) was used for β -tubulin detection. Protein S-nitrosylation levels were indicated as values after calibration with β -tubulin levels and normalization with the value in vehicle-treated slices without NOC7 application.

3. Results

3.1. PF-PTP and PF-LTP are impaired in the cerebellar slices from aged mice

We first examined whether PF-synaptic potentiation was impaired in cerebellar slices from aged mice. Excitatory postsynaptic currents (EPSCs) evoked by PF stimulation were recorded from PCs in the cerebellar slices from young (1-month old) or aged (20 to 24-month old) mice (Fig 1A). In the cerebellar slices from young mice, the amplitude of EPSCs at PF synapse (PF-EPSCs) was markedly potentiated and maintained stable at about 200% of the prestimulation level for at least 30 min after repetitive application of 60 burst stimulations (BSs, each BS = 5 pulses at 50 Hz) at 1 Hz (Fig. 1B, open

circles), as is observed in our previous study (Namiki et al., 2005). The early phase of the potentiation (1-2 min after the 60 BSs; post-tetanic potentiation (PTP)) was accompanied with a decrease in paired-pulse ratio (PPR) of PF-EPSC (Fig. 1C). Because inverse relationship between release probability and PPR had been observed in various synapses including PF synapses (Zucker and Regehr, 2002), the PF-PTP was at least partly dependent on presynaptic enhancement. On the other hand, the late phase of the potentiation (21-30 after 60 BSs; LTP) was not accompanied with changes in PPR, and indicated to be postsynaptic changes (Fig. 1C). In the cerebellar slices from aged mice, the amplitude of PF-EPSC was not markedly changed after repetitive application of 60 BSs, except for a slight increase shortly after the BSs (Fig. 1B, closed circles). The averaged PF-EPSC amplitudes during the 1-2 min period and 21-30 min period after the 60 BSs were 135.6 ± 12.5 % and 104.5 ± 0.6 % of the prestimulation levels, respectively, and these values were significantly lower than those in the young group (231.3 ± 22.6 % and 199.3 ± 7.4 %, respectively) (Fig. 1D, E). Thus, both PF-PTP and PF-LTP were revealed to be severely impaired in aged mice.

3.2. PF-PTP and PF-LTP are impaired in cerebellar slices from young mice pretreated with oxidative reagents

Our previous study demonstrated that both PF-PTP and PF-LTP were

dependent on NO signals, but not on sGC activity (Namiki et al., 2005). These facts indicated that PF-PTP and PF-LTP are dependent on the signals mediated by S-nitrosylation of cysteine residues. ROS also modifies cysteine residues and exert its action on cellular proteins. It has long been known that protein oxidation is enhanced in the brains of aged rodents (Cini and Moretti, 1995; Forster et al., 1996; Sohal et al., 1994; Vaishnav et al., 2007). Thus, it is highly possible that impairment of PF-PTP and PF-LTP in the aged cerebellum could be due to the blockade of protein S-nitrosylation by preceding protein oxidization by endogenous ROS. To test this hypothesis, effects of protein oxidation in the cerebellar slices from young mice with oxidizing agents on PF-PTP and PF-LTP were examined.

We first incubated acute cerebellar slices from young mice with H₂O₂, typical oxidizing agent which is known to induce functional changes in ionic channels (Annunziato et al., 2002; Cai and Sesti, 2009). In the cerebellar slices preincubated with H₂O₂, PF-PTP and PF-LTP were impaired in a dose-dependent manner (Suppl Fig. 1). In the cerebellar slices preincubated with 100 μ M H₂O₂, the average amplitude PF-EPSC during the 1-2 min period and 21-30 min period after the 60 BSs were 132.6 ± 11.0 % and 104.4 ± 3.0 % of the prestimulation levels, respectively, and these values were significantly lower than those in the vehicle-treated control group (237.0 ± 20.7 % and 193.7 ± 7.4 %, respectively) (Fig. 2).

To confirm the involvement of oxidation in impairment of the plasticities of PF synapses, we incubated the cerebellar slices from young mice with another

type of oxidizing agent, ChT. Both PF-PTP and PF-LTP were blocked after the preincubation of the cerebellar slices with ChT in a dose-dependent manner, too (Suppl Fig. 2). When the slices were pretreated with 10 μ M ChT, the average amplitude PF-EPSC during the 1-2 min period and 21-30 min period after the 60 BSs were 101.6 ± 5.1 % and 101.8 ± 1.0 % of the prestimulation levels, respectively, and these values were significantly lower than those in vehicle-treated control group (Fig. 2). Taken together, oxidation was revealed to be a factor exerting inhibitory effects on the induction of PF-PTP and PF-LTP in the cerebellar slices.

3.3. Hydrogen peroxide specifically inhibited signaling pathways essential for PF-synaptic plasticity

The results shown in figures 1-3 indicate that protein oxidation by endogenous ROS (in the cerebellar slices from aged mice) or oxidative reagents (in the cerebellar slices from young mice) impaired the induction of PF-PTP and PF-LTP possibly through inhibition of S-nitrosylation-mediated signals essential for the induction of these plasticity. However, it is still possible that protein oxidation indirectly inhibited these synaptic plasticities through impairment of synaptic function itself, because functional losses of ionic channels by oxidation had been reported in previous studies (Annunziato et al., 2002). To exclude these possibilities, we examined whether oxidation by the oxidizing agent (H_2O_2) affect basic electrophysiological properties of PF

synapses. We first examined effects of H₂O₂ treatment of the cerebellar slices from young mice on PPR. The ratio in H₂O₂-treated group was not significantly different from those in vehicle-treated control group in all intervals examined (10-300 ms) (Fig. 3A, B). The input-output relationships of the amplitudes of PF-EPSCs were not significantly different between H₂O₂-treated and vehicle-treated groups, too (Fig. 3C, D).

In some experiments, applications of oxidative reagents induce synaptic plasticity (Knapp and Klann, 2002). Thus, it is still possible that treatment with H₂O₂ induced synaptic potentiation during the incubation period, and occluded PF-PTP and PF-LTP induced by PF stimulation (60 BSs). Our results excluded this possibility because the amplitudes of PF-EPSCs during and 1-30 min after the application of H₂O₂ were not significantly different from those before application of the reagent (Fig. 4). The average amplitude PF-EPSC during the 7-9 min period and 30-39 min period after the onset of H₂O₂ application were $101.4 \pm 0.8 \%$ and $100.7 \pm 1.0 \%$ of the baseline levels (Fig. 4B, C). These results indicate that H₂O₂ application itself induce neither PF-PTP nor PF-LTP, and does not occlude synaptic plasticities induced by PF activity.

Application of H₂O₂ did not affect basic electrophysiological properties of PF synapse (Fig. 3) and did not occlude PF-PTP and PF-LTP (Fig. 4). Therefore, these results exclude the possibility that the impairments of PF-PTP and PF-LTP by oxidizing agents were the secondary effects of oxidation in the cerebellar slices, and therefore strongly indicate that

oxidation specifically inhibited signaling pathways essential for the induction of PF-PTP and PF-LTP.

3.4. Thiol residues are essential regulatory sites for PF-synaptic plasticities

As is described above, oxidation specifically impaired signaling pathways essential for the induction of the plasticities of PF synapse. Previous studies indicated that the PF-synaptic potentiation induced by repetitive stimulation of PF were dependent on signaling pathways mediated by protein S-nitrosylation by NO (Lev-Ram et al., 2002; Namiki et al., 2005). Thiol groups in cysteine residue are the target of S-nitrosylation of proteins by NO as well as the target of oxidation by endogenous ROS and oxidizing agents. Thus, it is highly possible that protein oxidation blocks the induction of PF-PTP and PF-LTP through the inhibition of protein S-nitrosylation in the cerebellar slices.

To test this hypothesis, we conducted several lines of experiments. We first examined whether thiol groups in cysteine residues were in fact essential for PF-PTP and PF-LTP. Preincubation of the cerebellar slices from young mice with 50 μ M N-ethyl-maleimide (NEM), a membrane-permeable thiol blocker, severely impaired both PF-PTP and PF-LTP (Fig. 5), indicating essential roles of thiol groups in PF-synaptic plasticities. Excess amounts of exogenous thiol-donors scavenge NO and thus inhibit S-nitrosylation of thiol groups in endogenous proteins. Therefore, we next examined effects of thiol

donors to further confirm the essential role of S-nitrosylation of thiol groups in PF-synaptic plasticities. When thiol donors, glutathione (reduced form; GSH) and N-acetyl-cysteine (NAC), were bathly applied, PF-LTP but not PF-PTP were severely impaired (Fig. 6). It is demonstrated that nitric oxide synthase is expressed in PF terminal (the axon of cerebellar granule cell) but not in Purkinje cell (Garthwaite and Boulton, 1995), and that PF-PTP was presynaptically induced (Fig. 1C). Because GSH and NAC are membrane-impermeable thiol donors, these drugs could scavenge the NO released into synaptic cleft only and impaired S-nitrosylation of thiol groups within Purkinje cells but not within PF terminal. Thus, bath application of GSH and NAC could specifically inhibit PF-LTP. Taken together, these results strongly indicate the essential roles of thiol groups in cysteine residues in PF-synaptic plasticity.

3.5. Oxidation impaired protein S-nitrosylation in the cerebellar slices from young mice

Finally, we examined effects of protein oxidation on the S-nitrosylation of proteins in the cerebellar slices from young mice. Incubation of the cerebellar slices from young mice with NO donor, NOC7 (300 μ M), resulted in the elevation of protein S-nitrosylation levels ($p < 0.001$; compare lanes 1 and 2 in Fig. 7A). However, preincubation of the slices with H₂O₂ severely impaired the NO-induced increase in protein S-nitrosylation levels ($p < 0.001$;

compare lanes 2 and 4 in Fig 7A), although pretreatment with H₂O₂ itself did not affect the levels before the NO treatment ($p > 0.05$; compare lanes 1 and 3 in Fig. 7A). The normalized values of protein S-nitrosylation levels with and without NOC7 application were 2.42 ± 0.03 and 1.00 ± 0.01 in the vehicle-pretreated control group (142 % increase) and 1.10 ± 0.02 and 0.94 ± 0.02 in the H₂O₂-pretreated group (16 % increase). Nitric oxide-induced increase in protein S-nitrosylation levels was severely impaired in the cerebellar slices from aged mice, when compared with young animals. The normalized values of protein S-nitrosylation levels with and without NOC7 application were 2.50 ± 0.10 and 1.00 ± 0.07 in the slices from young mice (150 % increase) and 1.52 ± 0.06 and 1.05 ± 0.01 in the slice from aged mice (47 % increase) (Fig. 7B). These biochemical data in Fig 5 correspond very well with the electrophysiological data in Figs 1-2: PF-PTP and PF-LTP as well as NO-induced increase in protein S-nitrosylation levels were severely impaired in the cerebellar slices from aged mice or in the cerebellar slices pretreated with oxidizing agents. Therefore, these results strongly indicate inhibitory action of protein oxidation on the induction of PF-synaptic plasticities through the impairment of protein S-nitrosylation by acute NO signal.

4. Discussion

The strong correlation between increasing age and the accumulation of

oxidative damage has largely supported the oxidative stress hypothesis of aging (Droge and Schipper, 2007; Serrano and Klann, 2004). In aged brain of rodents, protein oxidation is enhanced (Cini and Moretti, 1995; Forster et al., 1996; Sohal et al., 1994; Vaishnav et al., 2007) and behavioral deficits of aged animals are associated with increases in oxidative stress (Butterfield et al., 2006; Cantuti-Castelvetri et al., 2000; Carney et al., 1991; Forster et al., 1996; Fukui et al., 2001). These observations strongly indicate that oxidative stress is a primary factor inducing deficits in synaptic plasticity and memory in aged animals. However, how oxidative stress results in the impairment of synaptic plasticity and brain function are not well understood. Previous studies indicate that ROS modulate activity of protein kinase C, extracellular signal-related kinase, protein tyrosine kinases, protein phosphatase 2A and calcineurin (Klann and Thiels, 1999; Serrano and Klann, 2004). In these cases, oxidation affects synaptic plasticity through the modulation of enzymatic activities in non-competitive manners. In the present study, preincubation of cerebellar slices from young mice with oxidizing agents resulted in blockade of PF-synaptic plasticities as well as S-nitrosylation of proteins. Because PF-PTP and PF-LTP are indicated to be dependent on S-nitrosylation, the current study provides the first evidence that oxidation impaired synaptic plasticity through the inhibition of S-nitrosylation in a “competitive” manner.

Various studies demonstrate functional modulation of ionic channels by oxidation (Aizenmann et al. 1989; Annunziato et al., 2002; Cai and Sesti,

2009). In the present study, treatment of the cerebellar slices with oxidizing agents did not induce significant changes in basic electrophysiological properties of PF-EPSC (Fig. 3), although the treatment impaired PF-synaptic plasticity (Fig. 2). Probability of neurotransmitter release is dependent on the calcium influx through voltage-dependent calcium channels (VDCCs) at presynaptic terminals, and changes in the functions of AMPA-type glutamate receptors (AMPA-Rs) affect the amplitude of EPSC. Therefore, functions of ionic channels such as VDCCs and AMPARs should not be affected by the oxidizing agents, at least in our experimental condition. Moreover, these observations indicate that the signaling pathways essential for the induction of PF-synaptic plasticities are more sensitive to the oxidative stress than those ionic channels involved in the synaptic transmission (eg. VDCCs and AMPARs).

Although the present study demonstrated that oxidation impaired PF-synaptic plasticities through the inhibition of S-nitrosylation essential for the plasticity in a competitive manner, the target molecule(s) of the oxidation was not identified. Sixty BSs induced synaptic plasticities accompanied with presynaptic change (PF-PTP) as well as postsynaptic change (PF-LTP). In the cerebellar slices from the aged mice or pretreated with oxidizing agents (from the young mice), both PF-PTP and PF-LTP were abolished. These evidences suggest that a common target molecule(s) of the oxidation should be localized at both presynaptic and postsynaptic site. Alternatively, it is also possible that some distinct molecules involved in PF-synaptic plasticities

dependent on protein S-nitrosylation are localized at presynaptic and postsynaptic site and are targets of oxidation.

It is demonstrated in various studies that behavioral deficits of aged animals are associated with increases in oxidative stress. Because nitric oxide synthase are widely expressed in the nervous systems (Bredt and Snyder, 1994; Calabrese et al., 2007; Garthwaite and Boulton, 1995; Huang, 1997; Snyder, 1992), the results in the present study strongly indicate that many biological events including synaptic plasticity are dependent on protein S-nitrosylation by NO and impaired by oxidation in a competitive manner. Identification of cysteine residues in target molecules of S-nitrosylation as well as oxidation and development of drugs specifically reduce the oxidized cysteine residue will open a new avenue in the study of anti-aging for minimizing age-related decline in the brain function.

Conflict of interest

All authors report no actual or potential conflicts of interests.

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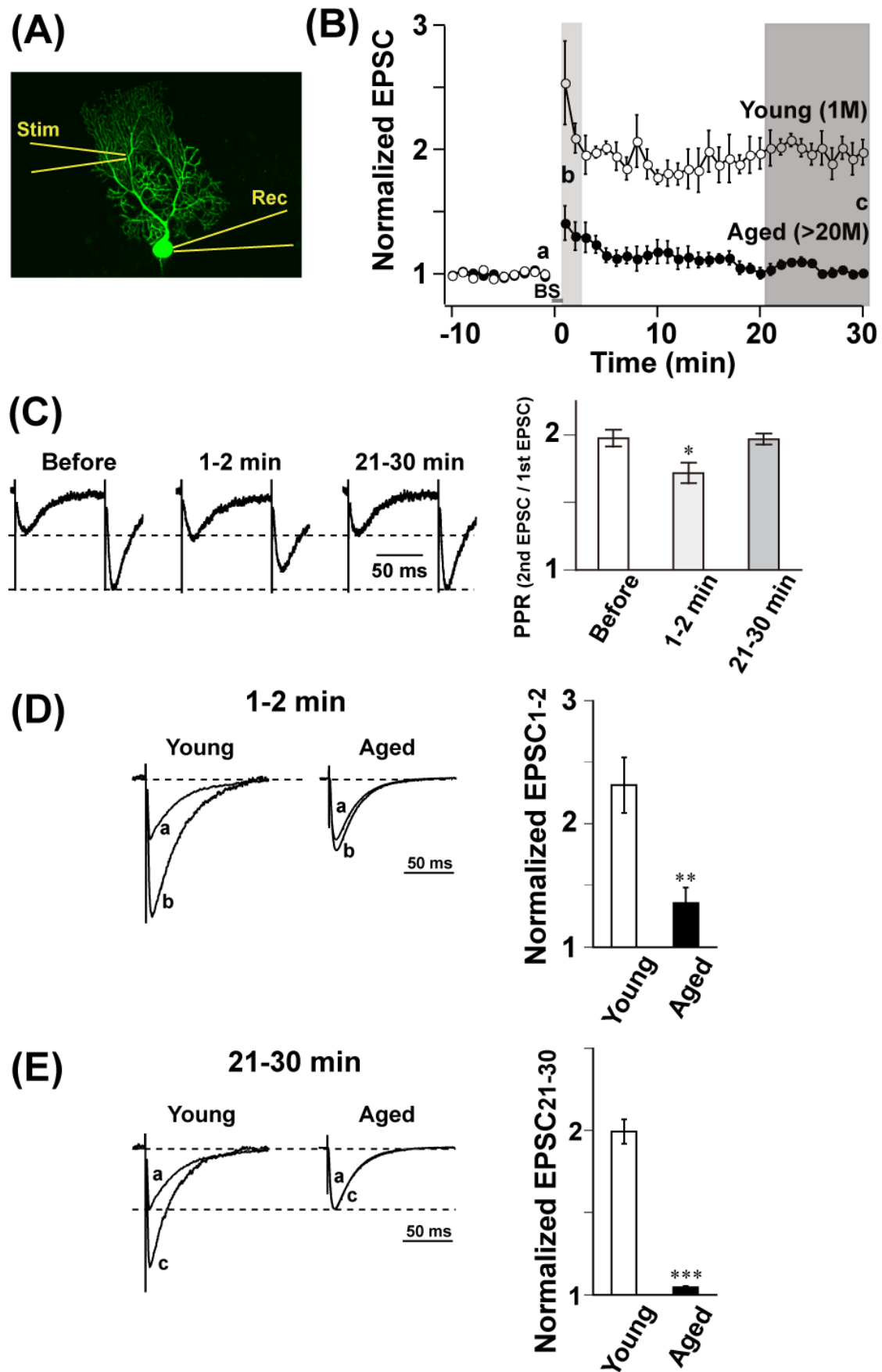
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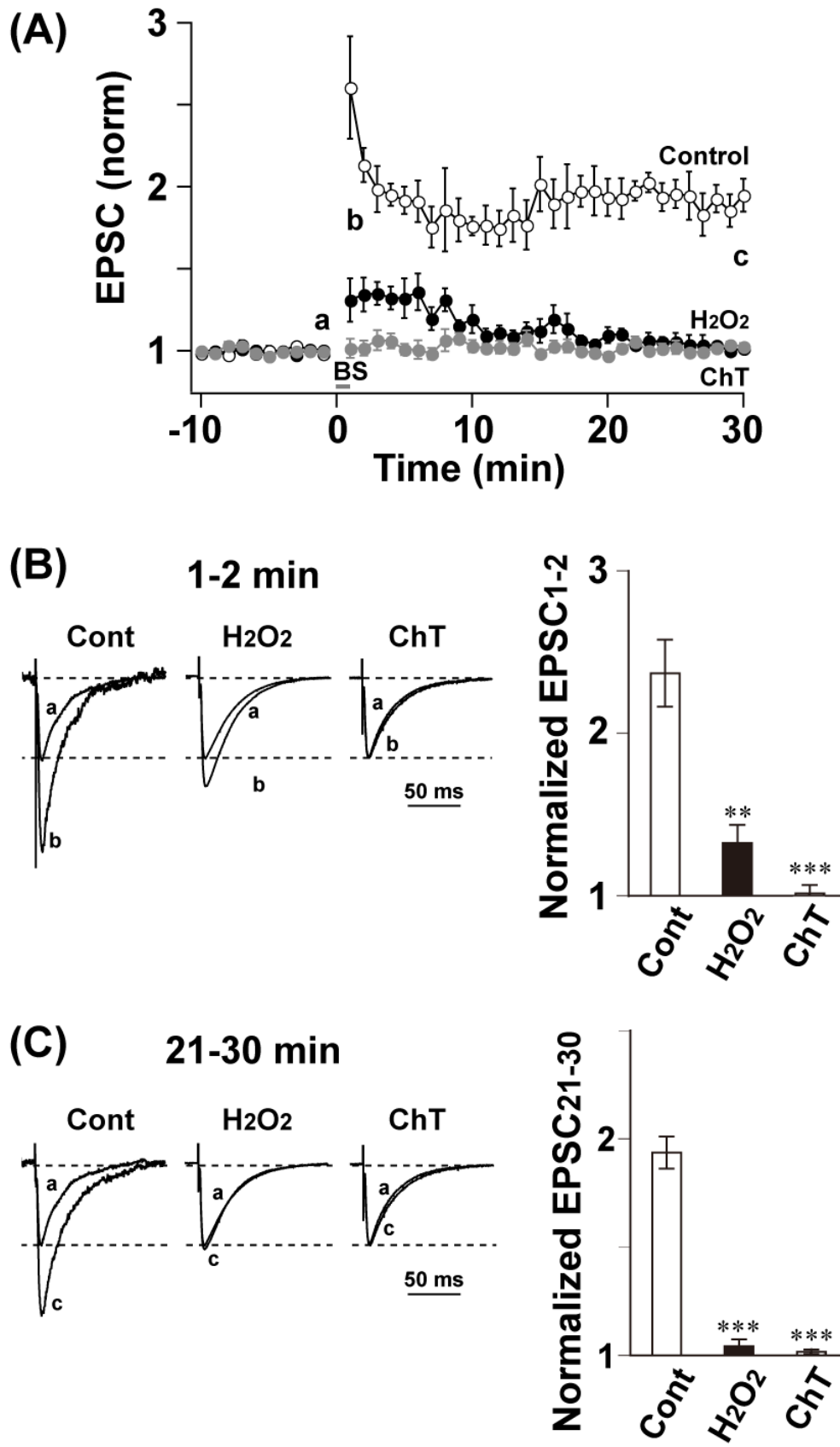
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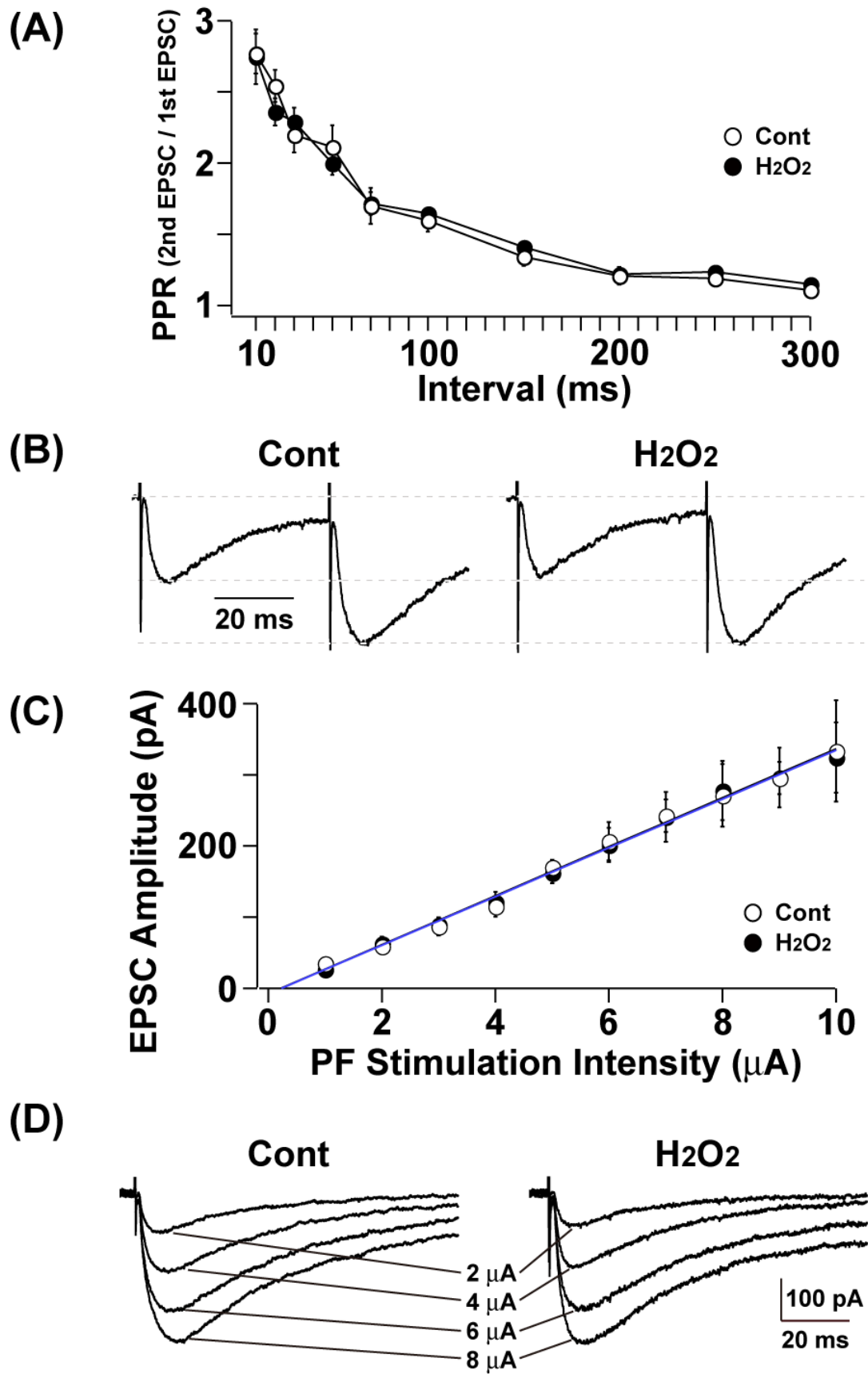
Kakizawa et al. Fig. 1

Fig. 1 Inhibition of PF-synaptic plasticity in aged mice. (A) Schematic image of electrophysiological recording from a PC in the cerebellar slice. Rec, recording pipette; Stim, stimulation pipette. (B) PTP and LTP were induced by 60 BSs repetitively applied at 1 Hz in the cerebellar slices from young mice ($n=6$), but not from aged mice ($n=5$). The amplitude was normalized by the mean value observed for 10 min before the BSs. (C) PPR was changed during the 1-2 min period after BSs (light-gray shadow in (B)), but not during 21-30 min period after BSs (dark-gray shadow in (B)) in the cerebellar slices from young mice. Representative traces (left) and average PPR values (right) before BSs (a) and 1-2 min (b) and 21-30 min (c) after BSs. Interpulse interval was 100 ms. $*p<0.05$, significantly different from the value before BSs. (D) PF-PTP was impaired in aged mice. Typical traces (left) and average amplitude (right) of PF-EPSC recorded from cerebellar slices from young and aged mice before (a) and 1-2 min after (b) the BSs. $**p<0.01$, significantly different from the value in young group. (E) PF-LTP was impaired in aged mice. Typical traces (left) and average amplitude (right) of PF-EPSC recorded from cerebellar slices from young and aged mice before (a) and 21-30 min after (c) BSs. $***p<0.001$, significantly different from the value in young group. All values are expressed as Mean + S.E.M.



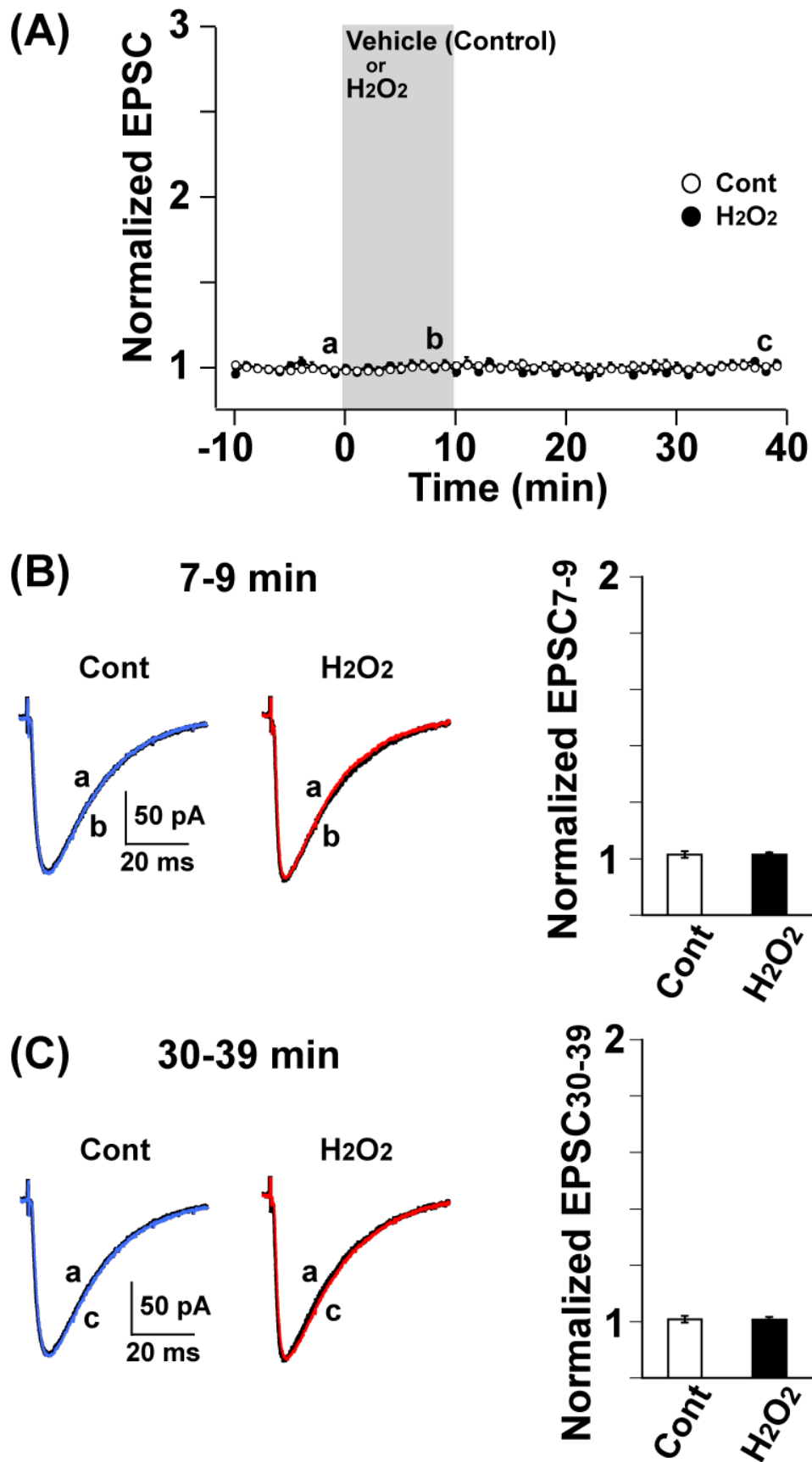
Kakizawa et al. Fig. 2

Fig. 2 Impairment of PF-synaptic plasticity by oxidizing agents. (A) PTP and LTP were induced by 60 BSs repetitively applied at 1 Hz in the cerebellar slices preincubated with vehicle (control, open circle; $n=6$), but not in the cerebellar slices preincubated with 100 μM H_2O_2 (closed black circle; $n=5$) or 10 μM ChT (closed gray circle; $n=5$). The amplitude was normalized by the mean value observed for 10 min before the BSs. (B) PF-PTP was impaired by oxidizing agents. Typical traces (left) and average amplitude (right) of PF-EPSC before (a) and 1-2 min after (b) the BSs. The cerebellar slices from young mice were treated with vehicle, H_2O_2 or ChT. $**p<0.01$, $***p<0.001$, significantly different from the value in control group. (C) PF-LTP was impaired by oxidizing agents. Typical traces (left) and average amplitude (right) of PF-EPSC before (a) and 21-30 min after (c) the BSs. The cerebellar slices from young mice were treated with vehicle or H_2O_2 or ChT. $***p<0.001$, significantly different from the value in control group. All values are expressed as Mean \pm S.E.M.



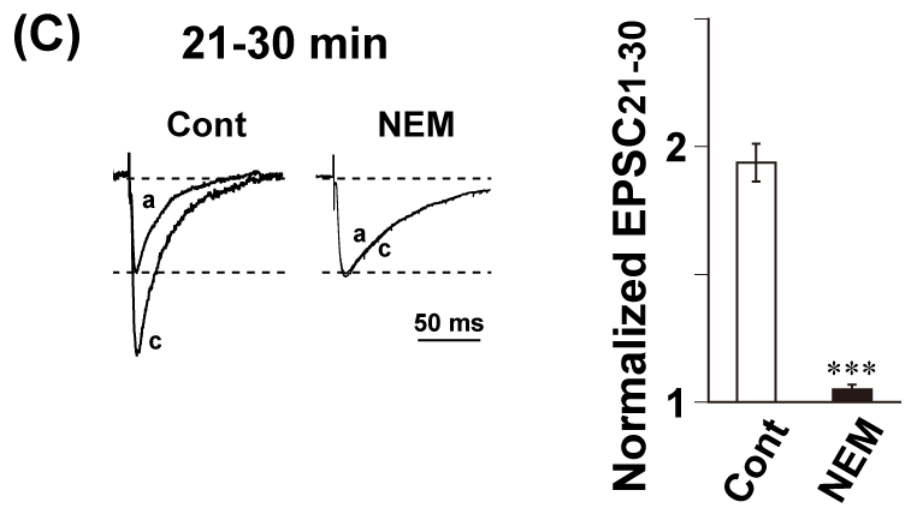
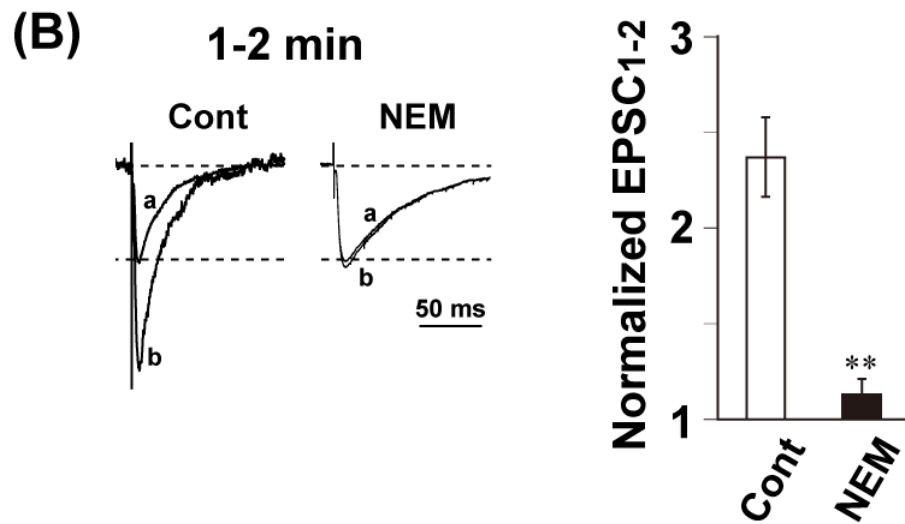
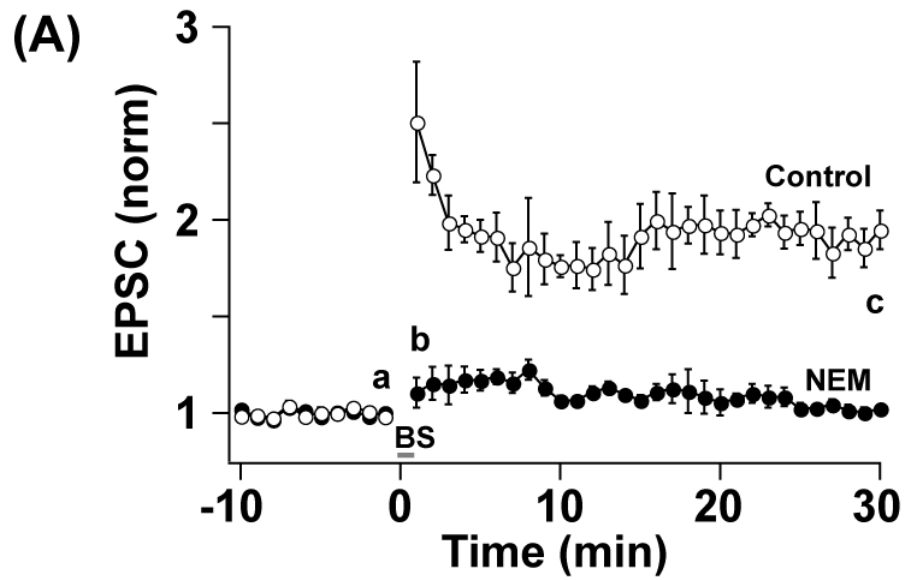
Kakizawa et al. Fig. 3

Fig. 3 Basic electrophysiological properties of PF-EPSC are unaffected by H₂O₂. (A) Paired-pulse ratio of PF-EPSC in H₂O₂-treated group (n=9) was not significantly different from those in vehicle-treated group (n=10). (B) Typical current responses of PF synapse to paired-pulse stimulation (interpulse interval = 50 ms) from cerebellar slices preincubated with vehicle and H₂O₂. (C) Input-output relation in H₂O₂-treated group (n=5) was not significantly different from those in vehicle-treated group (n=8). First regression lines of vehicle- and H₂O₂-treated groups were shown as black and blue lines, respectively. (D) Typical current responses of PF synapse evoked by stimulation with increasing intensities from 2 to 8 μ A in vehicle- and H₂O₂-treated groups. All values are expressed as Mean \pm S.E.M.



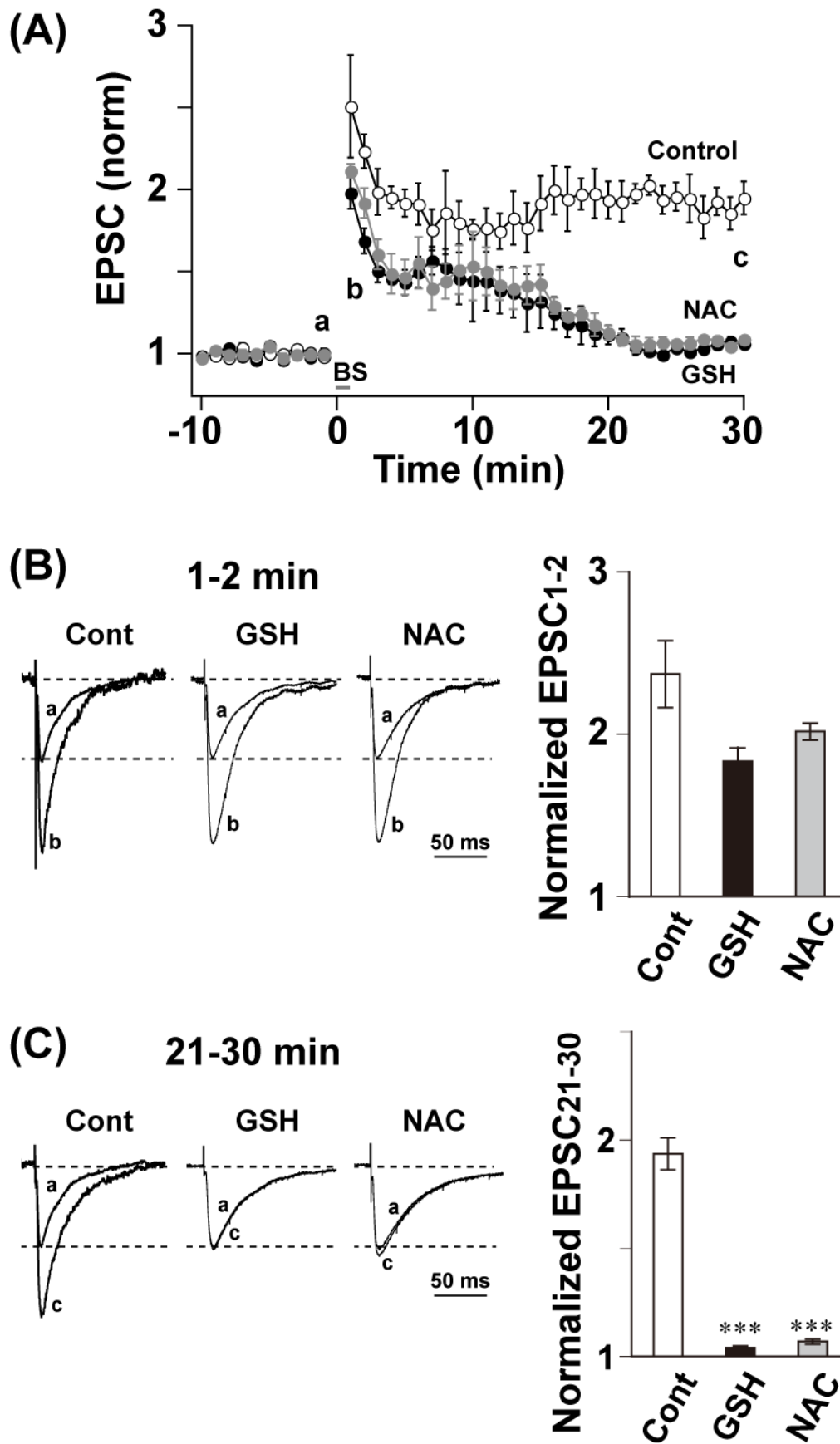
Kakizawa et al. Fig. 4

Fig. 4 Application of H₂O₂ does not induce potentiation at PF synapse. (A) The amplitude of PF-EPSC before, during (shadow) and after bath application of vehicle (n=5) or H₂O₂ (n=4) for 10 min. The amplitude was normalized by the mean value before H₂O₂ application. (B) Typical traces (left) and average amplitude (right) of PF-EPSC recorded from cerebellar slices before (1, black lines) and 7-9 min after (2) the onset of vehicle (blue) or H₂O₂ (red) application. (C) Typical traces (left) and average amplitude (right) of PF-EPSC recorded from cerebellar slices before (1, black lines) and 30-39 min after (3) the onset of vehicle (blue) or H₂O₂ (red) application. All values are expressed as Mean \pm S.E.M.



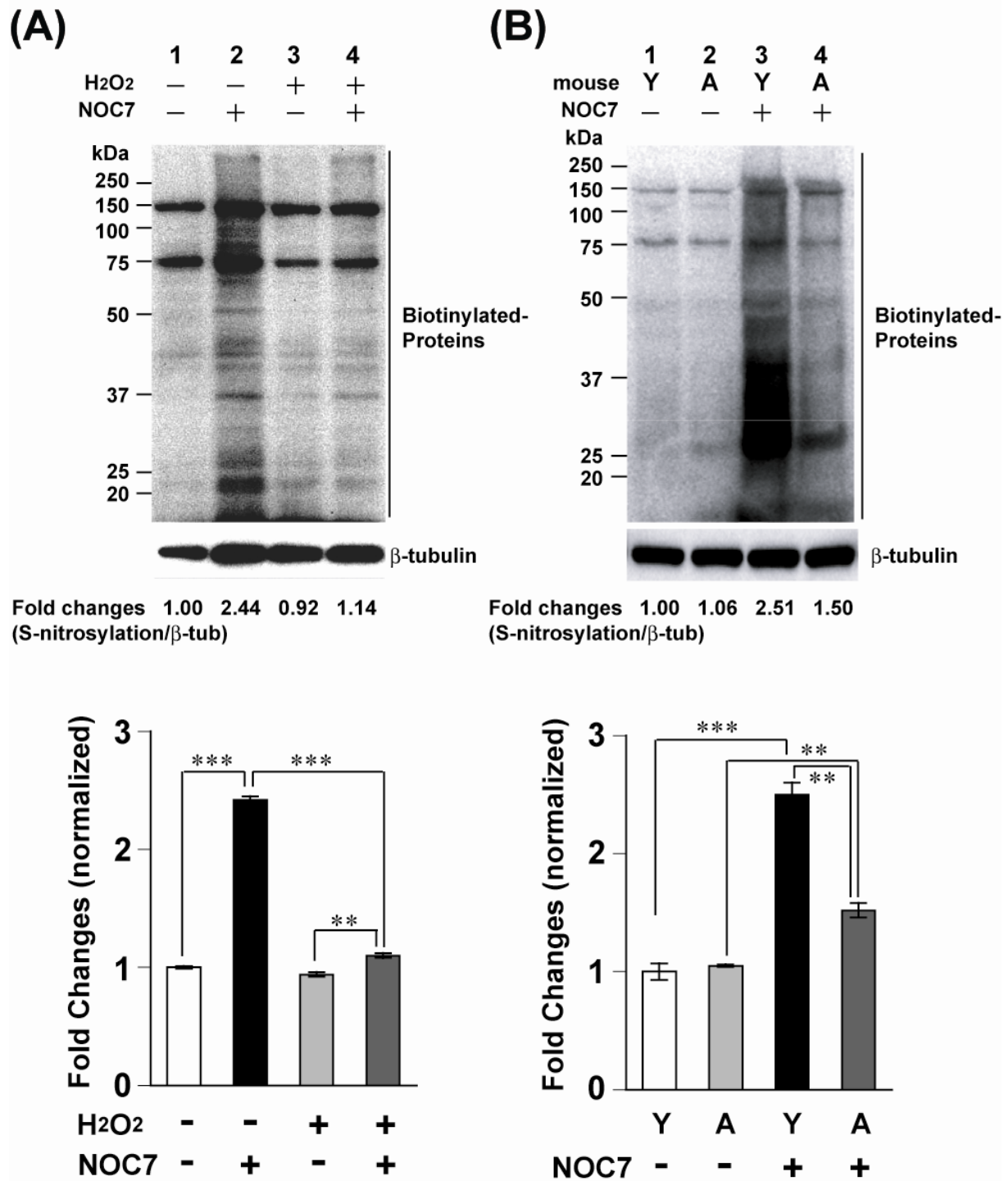
Kakizawa et al. Fig. 5

Fig. 5. Effects of membrane-permeable thiol blocker, N-ethyl-maleimide (NEM) on PF-PTP and PF-LTP. (A) The amplitude of PF-EPSC before, during (bar) and after 60 BSs at 1 Hz in the cerebellar slices preincubated with vehicle (control, open circle; n=6) and 50 μ M (closed circle; n=5) NEM for 5 min. The amplitude was normalized by the mean value observed for 10 min before the BSs. (B-C) Typical traces and average amplitude of PF-EPSC 1-2 (B) and 21-30 min (C) after the BSs in the cerebellar slices pretreated with vehicle (control) and 50 μ M NEM for 5 min. ** $p<0.01$, *** $p<0.001$, significantly different from the value in control group. All values are expressed as Mean \pm S.E.M.



Kakizawa et al. Fig. 6

Fig. 6. Effects of membrane-impermeable thiol donor, glutathione (reduced form; GSH) and N-acetyl-cysteine (NAC) on PF-PTP and PF-LTP. (A) The amplitude of PF-EPSC before, during (gray bar) and after 60 BSs at 1 Hz in the cerebellar slices in the presence of vehicle (control, open circle; n=6), 500 μ M GSH (closed black circle; n=5) and 500 μ M NAC (closed gray circle; n=5). The amplitude was normalized by the mean value observed for 10 min before the BSs. (B-C) Typical traces and average amplitude of PF-EPSC 1-2 (B) and 21-30 min (C) after the BSs in the cerebellar slices in the presence of vehicle, 500 μ M GSH and 500 μ M NAC. *** p <0.001, significantly different from the value in control group.



Kakizawa et al. Fig. 7

Fig. 7. NO-induced protein S-nitrosylation were impaired in the cerebellar slices from aged mice or slices pretreated with oxidizing agent. The overall protein S-nitrosylation in cerebellar slices treated with NOC7 was resolved by the biotin-switch assay. (A) (upper) Effects of H₂O₂ on NO-induced protein S-nitrosylation in the cerebellar slices from young mice. Lane 1, vehicle-pretreated cerebellar slice; Lane 2, cerebellar slice treated with 300 μ M NOC7 for 10 min after the pretreatment with vehicle for 10 min; Lane 3, H₂O₂-pretreated cerebellar slice; Lane 4, NOC7-treated cerebellar slice after the pretreatment with H₂O₂ for 10 min. Molecular weights of marker proteins are given to the left. The values in the bottom are overall protein S-nitrosylation levels, calibrated with β -tubulin levels and then normalized with the value in vehicle-pretreated group (lane 1). (lower) Summary of the fold changes in S-nitrosylation levels. Mean \pm S.E.M. (n=3). ** p < 0.01; *** p < 0.001. (B) (upper) NO-induced protein S-nitrosylation in the cerebellar slices from young or aged mice. Lane 1, cerebellar slice from young mice treated with vehicle for 10 min; Lane 2, cerebellar slice from aged mice treated with vehicle for 10 min; Lane 3, cerebellar slice from young mice treated with 300 μ M NOC7 for 10 min; Lane 4, cerebellar slice from aged mice treated with 300 μ M NOC7 for 10 min. Molecular weights of marker proteins are given to the left. The values in the bottom are overall protein S-nitrosylation levels, calibrated with β -tubulin levels and then normalized with the value in vehicle-pretreated slices from young mice (lane 1). (lower) Summary of the fold changes in S-nitrosylation levels. Mean \pm S.E.M. (n=3).

**** $p < 0.01$; *** $p < 0.001$.**